THE INHIBITION BY N,N´-DICYCLOHEXYLCARBODIIMIDE OF TRANSLOCATION OF AUXIN IN INTACT BEAN SEEDLINGS AND ITS REVERSAL BY GIBBERELLIN A_3

By

CHRISTOPHER WILLIAM CORBETT

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Oklahoma State University

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Thesis Approved:

Thesis Adviser

Dean of Graduate College

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ABBREVIATIONS

ADP	adenosine diphosphate
ancymidol	α-cyclopropyl-α-(4-methoxyphenyl)-4-pyrimidine-methanol
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
DCCD	N,N´-dicyclohexylcarbodiimide
DPX1840	3,3a-dihydro-2-(p-methoxyphenyl)-8H-pyrazolo(5,1-a) isoindol-8-one
EDTA	ethylenediamine tetraacetic acid
ethephon	2,chloroethyl-phosphonic acid
GA	gibberellin, A_3
IAA	indole-3-acetic acid
morphactins	mixture, methyl 2-chloro-9-hydroxyfluorene-9-carboxylate; methyl 9-hydroxyfluorene-9-carboxylate; methyl 2,7-dichloro-9-hydroxyfluorene-9-carboxylate.
PCIB	p-chlorophenoxyisobutyric acid
Pi	inorganic phosphate
PPO	2,5-diphenyloxazole
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
TIBA	triiodobenzoic acid

CHAPTER I

INTRODUCTION

There are presently two prevailing theories of how auxin is translocated in plants. The two are not mutually exclusive. The first and oldest is referred to as "polar transport". This theory holds that auxin is produced in apical regions of the plant and is transported basipetally away from the apex in living but non-vascular tissue at rates of 0.5 to 2.0 cm/hr (Goldsmith et al., 1974). However, most studies of polar transport have used coleoptile or stem sections, thus effectively destroying the integrity of the phloem. Certain workers have begun to investigate the process of polar transport in intact plants by applying exogenous auxin to apical buds (Morris and Kadir, 1972, Morris et al., 1973, Bonnemain, 1971, in Morris and Kadir, 1972). These studies have reinforced the validity of the polar transport theory as a means of auxin movement from apical regions in living, non-vascular tissue at low velocities. Transport of this auxin from cell to cell by a protein carrier in the plasmalemma has growing support (Osborne and Mullins, 1969, Rubery and Sheldrake, 1974, Gaither and Abeles, 1975). Others, however, contend that this movement is due to pH and electrical potential gradients and not to a carrier (Raven, 1975).

Evidence has been accumulating that supports the second theory of auxin translocation. In this process, auxin is translocated long

distances in the phloem and at a much more rapid rate (16.0-24.0 cm/hr) than polar transport (Little and Blackman, 1963, Morris and Kadir, 1972, Morris et al., 1973, Long and Basler, 1973, Goldsmith et al., 1974 and Long and Basler, 1974). Naturally, this type of movement would not be detected in excised plant parts. Only when intact plants are used is auxin movement in the phloem observed.

The mechanisms of auxin movement in polar transport and vascular transport appear to be different. Compounds such as TIBA, PCIB, DPX1840 and morphactins are known to inhibit basipetal polar movement (Krelle and Libbert, 1967, Goldsmith, 1968, 1969, Morris et al., 1973, Gaither and Abeles, 1975) but they do not tend to inhibit movement of auxin occurring in the phloem (Morris et al., 1973, Long and Basler, 1973, Goldsmith et al., 1974, Basler, 1977).

Even if it has been established that there are two types of auxin movement in plants, the question still remains as to which is more important in the growth and development of the plant. There is evidence that non-vascular transport is important in such developmental processes as geotropism, phototropism and vascular tissue differentiation (Gaither and Abeles, 1975 and Sachs, 1975). An important question relates to the site of synthesis of auxin and how it gets to the meristematic regions where it promotes cell division and cell expansion. We must keep in mind that the very definition of a hormone includes that it is translocated from the site of synthesis to the site of action. Likely sites of synthesis of auxin are maturing and senescing parts of the plant such as leaves and stems, not meristematic areas such as buds (see review by Sheldrake, 1973). The precursor of the auxin indoleacetic acid is the amino acid tryptophan. It is

reasonable to assume that the most abundant source of tryptophan for auxin production would be regions of the plant such as the maturing xylem of leaves and stems where autolysis of proteins is occurring and not areas of meristematic activity where large amounts of tryptophan would be needed for synthesis of proteins. If indeed the former are primary regions of auxin synthesis, a logical pathway for translocation of the auxin to its site of activity would seem to be the phloem.

It is obvious that more attention must be given to the process of auxin translocation through the vascular tissues. A number of environmental factors such as temperature, humidity, salts, and water stress and physiological factors such as abscisic acid, gibberellic acid, and growth inhibitors have been found to affect auxin translocation in vascular tissue (Pallas, 1960, Basler et al., 1961, Long and Basler, 1973, Basler and Slife, 1974, Long and Basler, 1974, Basler, 1977). All of these are factors which typically affect the growth and development of the plant. It would seem likely that there is a connection between growth and auxin translocation.

Auxin is known to be a factor in a number of developmental processes in the plant. One of the most widely studied is its action on cell elongation in tropisms and in growth in general. The most widely held theory of how auxin causes elongation is the "proton extrusion" theory (Jacobs and Ray, 1976 and Cleland, 1976). Somehow auxin causes protons to be excreted from the cell cytoplasm to the free space around the cell wall. As the pH outside of the cell is reduced, enzymes which can cause loosening of the cell wall are activated. How does auxin cause this? There is some evidence to indicate that "auxin transport is auxin action", i.e., that the site of auxin transport may

be the site of auxin action and that the two may occur concomitantly, at least in certain cases (Hertel and Leopold, 1963, Rayle et al., 1969, Gaither and Abeles, 1975). This does not necessarily imply that the carrier for uptake into the phloem is the same carrier which causes uptake of auxin into the site of action. Long distance translocation and cell transport are probably two different processes but the membrane carriers may be related since both are involved in uptake.

One possible mode of action of auxin has been hypothesized to involve an adenosine triphosphatase bound to the plasma membrane (Hager et al., 1971). In this mechanism, auxin would activate an ATPase which would require ATP as an energy source to pump protons to the other side of the membrane. Plasma membrane-bound ATPases have been found in plants (Lai and Thompson, 1971 and Hodges et al., 1972). Inhibition by darkness of rapid auxin transport out of leaves has often been attributed to the fact that auxin in phloem flows along with assimilates and that darkness reduces assimilate production and flow. If an ATPase that requires ATP is involved however, the reduced transport of auxin may be due to a deficiency of ATP as a result of less light-induced oxidative phosphorylation.

Another indication that an ATPase may be involved is the inhibition of natural and auxin-induced elongation of cucumber hypocotyls by DCCD (Katsumi, 1976). DCCD has been shown to be an inhibitor of membrane-bound ATPases (Harold et al., 1969). Although all the evidence to date indicates that DCCD is a specific inhibitor of ATPase, this cannot be assumed to be universally true. The inhibition observed by Katsumi was irreversible but normal elongation

could be retained if gibberellic acid (GA) was added along with the DCCD treatment.

Therefore, in keeping with the assumption that auxin action and auxin transport and translocation are closely related, the present study will attempt to determine whether or not DCCD affects translocation of auxin and whether or not GA can reverse the effects, if any, of DCCD. Increasing our knowledge of the action of auxin, an important plant hormone, could aid in our understanding of the overall development of the plant.

CHAPTER II

MATERIALS AND METHODS

Bush beans (Phaseolus vulgaris L. cv. Stringless Green Pod) were germinated and incubated in perlite moistened with half-strength Hoagland's nutrient solution (Appendix A, Hoagland and Arnon, 1950) for 5 days at 31 C under continuous fluorescent light of 5.4 klux. The seedlings were then transplanted to amber glass jars containing 400 ml aerated half-strength Hoagland's solution and grown for 4 days prior to treatment in a growth chamber at 14 hr, 32 C, 19.9 klux days and 10 hr, 26 C nights with relative humidity ranging from 70 to 90%. Approximately 24 hr prior to treatment, the plants were transferred to fresh half-strength Hoagland's solution and were returned to the growth chamber. The fresh solution had been pre-aerated for at least 20 min. Five ml of Fe-EDTA solution (Appendix B, Steiner and Van Winden, 1970) was added to every 2 l of half-strength Hoagland's nutrient solution.

The plants were treated by injecting 1 µl containing either 0.5 µg 2,4,5-T-1-^{14}C (54.0 mCi/mmole) or 0.165 µg IAA-1-^{14}C (52.0 mCi/mmole) into the pith of the stem at the cotyledonary node to a point 1 cm below the node with a 1 µl syringe. The treatment chemicals were dissolved in 95% ethanol.

Other treatment chemicals injected into the stem in the same solution as the auxin were GA, DCCD or unlabeled 2,4,5-T. The

unlabeled 2,4,5-T was used when more than 0.5 µg 2,4,5-T was used for a treatment.

Eight replications were used for each treatment and plants were completely randomized within the growth chamber. Plants were treated at the fourth hour of the light period and were harvested 4 hr after treatment. Plants were divided into young shoots including all tissue above the primary leaves, primary leaves including petioles, epicotyl including all tissue 0.5 cm above the cotyledonary node to the primary leaf node, treated area including all stem tissue from 0.5 cm above the cotyledonary node to 2.0 cm below the node, hypocotyl including the remainder of the stem to the roots and finally the roots. The plant parts were freeze-dried, weighed and homogenized in 5.0 ml of 95% ethanol (or 10.0 ml for primary leaves) by a Brinkmann Polytron high-speed homogenizer. One-half ml aliquots (or 0.2 ml for primary leaves) of the homogenate were assayed for radioactivity by a Beckman LS-100 Liquid Scintillation System. Five ml aliquots of the nutrient solution were removed, freeze-dried, reconstituted with scintillation fluid (Appendix C) and assayed for radioactivity. Statistical analyses were standard F tests and Duncan's new multiple range test (Steel and Torrie, 1960).

Radiochemical purity of the 2,4,5-T-1-^{14}C and IAA-1-^{14}C had been previously determined by ascending paper chromatography in butanol: acetone:water (5:3:3, v/v/v) followed by scanning. Purity always exceeded 95%. These auxins were purchased from the Radiochemical Centre, Amersham, England. The unlabeled 2,4,5-T was purified by repeated recrystallization from benzene and 95% ethanol. The GA was

approximately 90% GA and was from the Sigma Chemical Company. The DCCD was from the K&K Labs of Cleveland, Ohio.

CHAPTER III

RESULTS

Interaction of GA and Different Levels of DCCD on Translocation of 2,4,5-T

DCCD was found to be very inhibitory to the acropetal and basipetal translocation of the auxin 2,4,5-T. The effects of 2,4,5-T injected alone or with three levels of DCCD in the presence or absence of GA are shown in Figures 1 and 2. Low levels of DCCD (2.0 µg) reduced acropetal auxin translocation to the young shoots to nearly a third of the control (Figure 1A) while it was almost totally inhibited by higher levels (6.0 and 20.0 µg). When 20.0 µg of GA was injected along with auxin and DCCD, significant maintenance of the normal movement of auxin to the young shoots was observed, especially at the lowest level of DCCD. GA added at the highest DCCD level only slightly nullified the DCCD inhibition. The GA at the 6.0 µg DCCD level caused restoration of movement of 2,4,5-T to about 40 percent of the normal.

DCCD inhibition of 2,4,5-T translocation to the primary leaves was extensive but not as extreme as that seen in the young shoots (Figure 1B). Low levels of DCCD reduced accumulation in primary leaves by a third while high levels reduced it by about two-thirds. Addition of GA to the 2.0 µg and 6.0 µg levels of DCCD caused moderate restoration of auxin translocation but at the highest level of DCCD, a very high degree of GA action on the DCCD activity was seen. This is quite

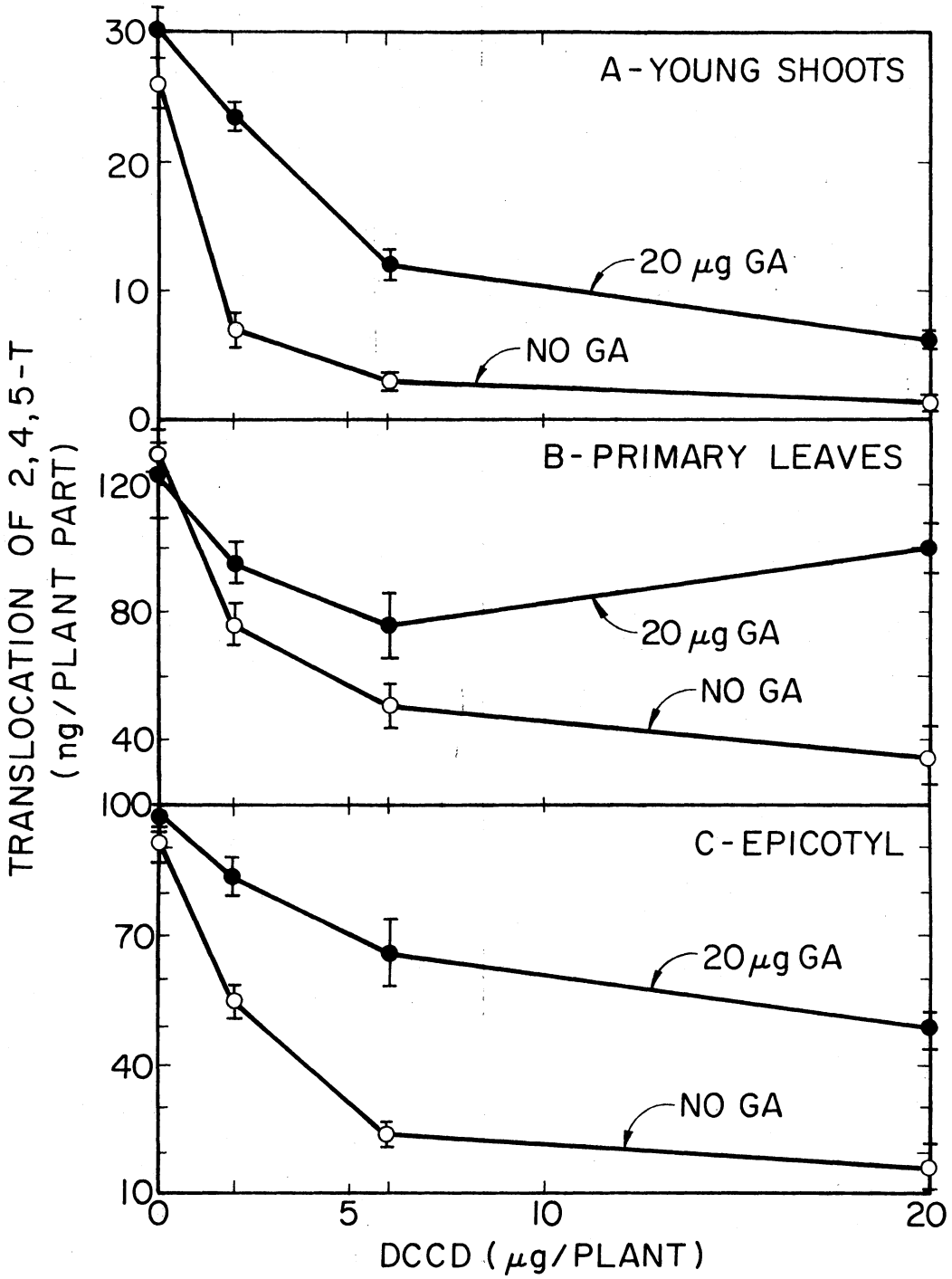

Figure 1. Effects of DCCD with or without GA on translocation of 2,4,5-T in (A) young shoots, (B) primary leaves and (C) epicotyl. Levels of 2,4,5-T and GA were, respectively, 0.5 μg and 20.0 μg per plant. Brackets are standard errors of the mean.

different from the slight reversal by GA of inhibition of translocation of 2,4,5-T to young shoots by high levels of DCCD.

DCCD also greatly reduced the translocation of auxin to the epicotyl especially at the 6.0 and 20.0 µg treatments (Figure 1C). In the case of the epicotyl, GA induced, overall, a higher degree of maintenance of translocation capability than in young shoots or primary leaves.

As would be expected from the above results, DCCD tended to inhibit translocation of auxin out of the treated area around the cotyledonary node (Figure 2A). It is interesting to note here that inhibition of movement of auxin out by 20.0 µg DCCD is less than double that caused by 2.0 µg DCCD, even though the former level is ten-fold that of the latter. The 20.0 µg of GA reduced by about one-third, the inhibition of movement of auxin out of the treated area at each level of DCCD.

DCCD caused a reduction in basipetal translocation of 2,4,5-T into the hypocotyl (Figure 2B). In this case, however, the GA added to the 2.0 µg DCCD treatment restored the auxin movement to 100 percent of the control and was effective in reducing the effects of 6.0 µg of DCCD. On the other hand, GA was only slightly effective in reversing the inhibition at the 20.0 µg level of DCCD.

The values for the roots and the nutrient solutions were combined (Figure 2C). High levels of DCCD were only slightly more effective in reducing movement of auxin into the roots and nutrient solution than low levels. Yet this still resulted in more than 50 percent inhibition of the normal value. GA had no effect on nullifying the inhibition of the highest level of DCCD and only a slight effect on the next highest

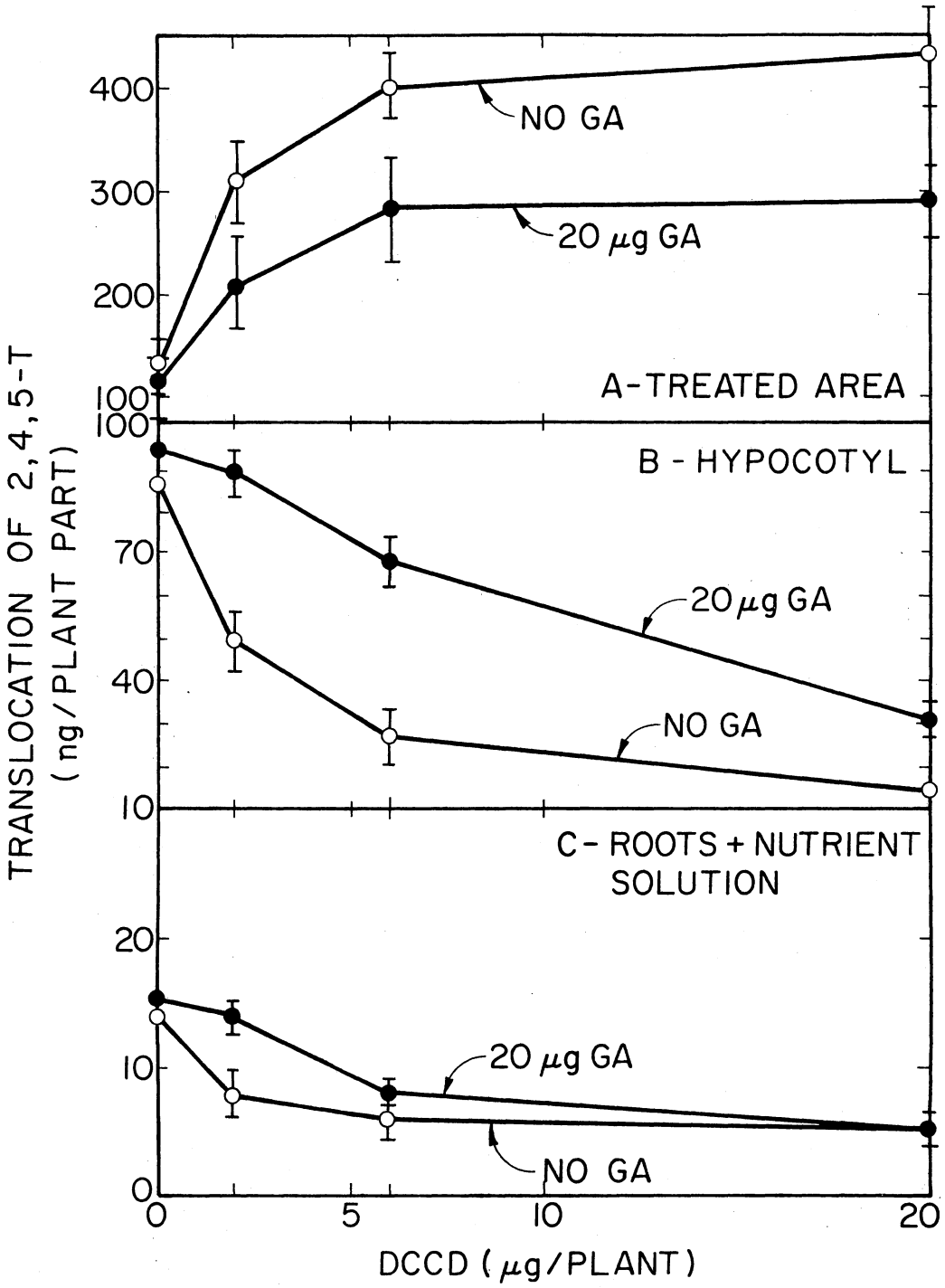

Figure 2. Effects of DCCD with or without GA on translocation of 2,4,5-T in (A) treated area, (B) hypocotyl and (C) roots and nutrient solution. Levels of 2,4,5-T and GA were, respectively, 0.5 μg and 20.0 μg per plant. Brackets are standard errors of the mean.

level. This contrasts with the almost 100 percent nullification by GA of the 2.0 µg DCCD inhibition.

Effects of Various Levels of GA on DCCD Inhibition of Auxin Translocation

Four levels of GA, from 2.0 µg to 40.0 µg were used to investigate the range of GA effective in nullifying the inhibition of auxin translocation by DCCD. A single level of 6.0 µg of DCCD was used with 0.5 µg of 2,4,5-T. The control had auxin but no GA or DCCD. The two lowest levels of GA, 2.0 µg and 6.0 µg, caused no reversal of the inhibition caused by DCCD in the young shoots (Figure 3A). Twenty µg of GA restored translocation to 35 percent of normal while the highest level of GA induced an almost complete return to the levels of auxin movement seen in the control.

This same pattern was seen in the primary leaves (Figure 3A). Twenty µg of GA caused a restoration of translocation to 60 percent of normal from a maximum inhibition of 35 percent of normal. The highest level of GA (40 µg) returned the auxin translocation nearly to normal.

The patterns of GA reversal of DCCD inhibition of auxin movement to the epicotyl and hypocotyl were almost identical (Figure 3B). In both plant parts, the two lowest levels of GA had no effect on the DCCD inhibition. The 20.0 µg level of GA returned auxin translocation to about 75 percent of the control. The 40.0 µg level returned the movement of auxin to the epicotyl to the control level but it induced movement to the hypocotyl to 20 percent above the control.

Inhibition of auxin translocation out of the treated area followed the trend seen in the previous plant parts (Figure 4A). The two lowest

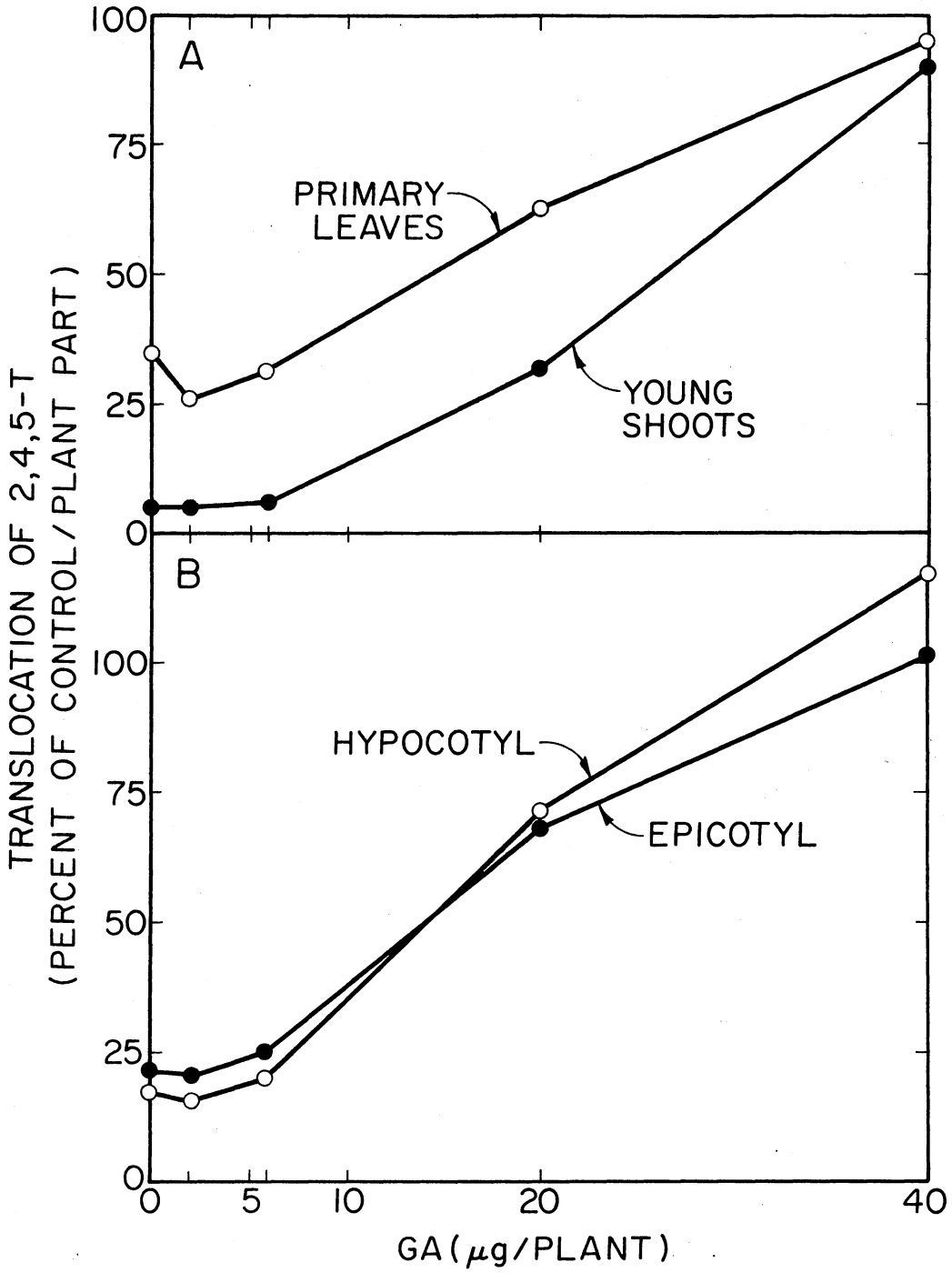

Figure 3. Influence of concentration of GA on reversal of DCCD inhibition of auxin translocation in (A) young shoots and primary leaves and (B) epicotyl and hypocotyl. Values on the ordinate are percent of control. Levels of 2,4,5-T and DCCD were, respectively, 0.5 μg and 6.0 μg per plant.

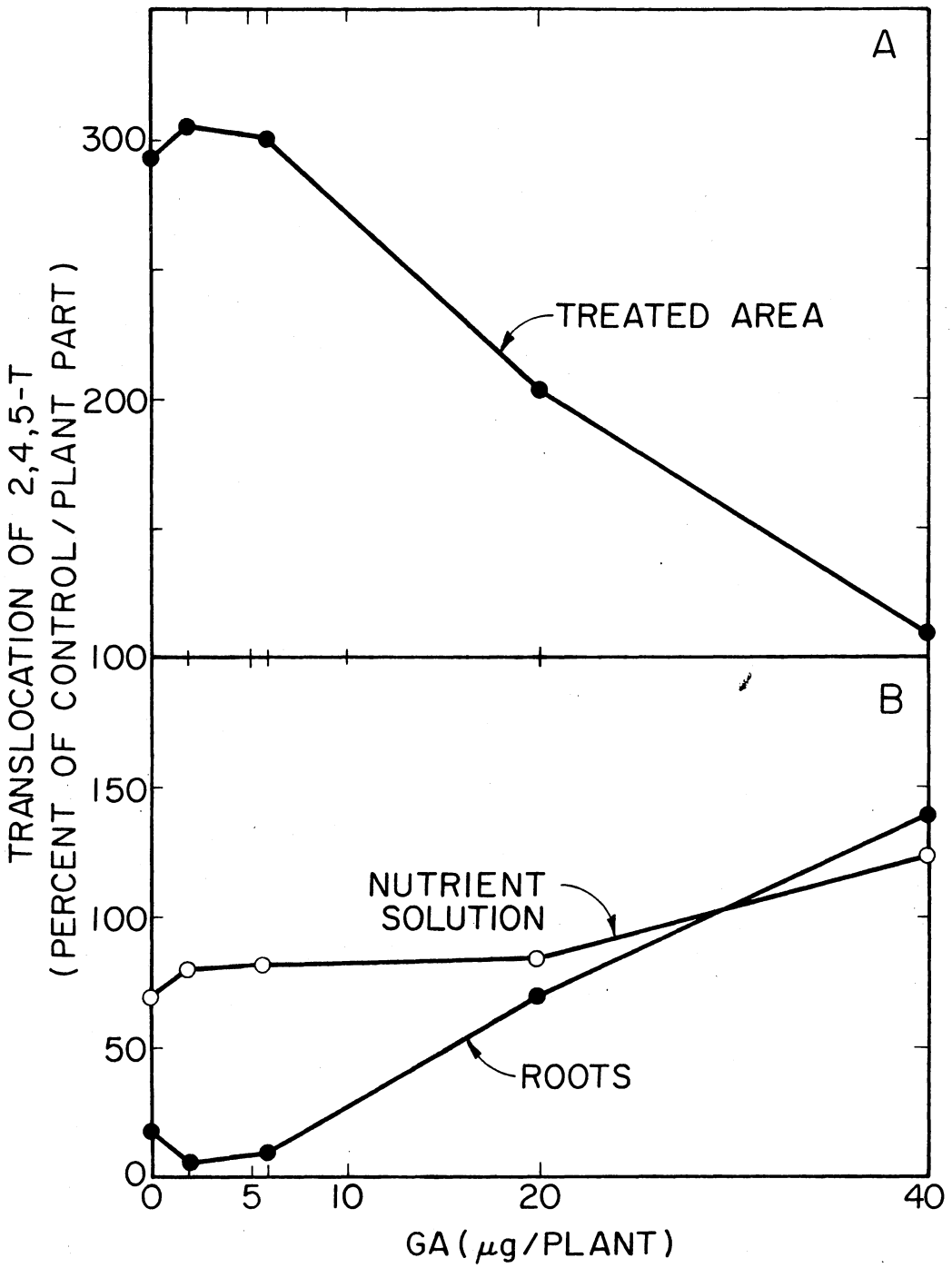

Figure 4. Influence of concentration of GA on reversal of DCCD inhibition of auxin translocation in (A) treated area and (B) roots and nutrient solution. Values on the ordinate are percent of control. Levels of 2,4,5-T and DCCD were, respectively, 0.5 μg and 6.0 μg per plant.

levels of GA caused no significant movement out of the treated area. The 20.0 µg GA treatment caused twice as much auxin to move out as the maximally inhibited plants. The highest GA level restored movement out of the treated area to the level normally seen in the control.

The 2.0 µg and 6.0 µg levels of GA provided no alleviation of the state of DCCD inhibition of auxin translocation to the roots, which was about 15 percent of the control (Figure 4B). The 20.0 µg treatment caused a return of auxin translocation to about 70 percent of normal. The highest level of GA caused a significant increase in auxin translocation of 40 percent above the control.

The 6.0 µg of DCCD caused only a relatively small inhibition (about 70 percent of control) of movement of 2,4,5-T into the nutrient solution (Figure 4B). This is much less inhibition of translocation than was seen in any of the plant parts, except, of course, the treated area. The three lowest levels of GA all caused a nonsignificant restoration of translocation. The highest level of GA induced translocation of auxin into the nutrient solution to about 25 percent above the level of the control.

The values in Figures 3 and 4 are percentages of the control. Actual values of controls of each plant part are: young shoots-49.7 ng, primary leaves-281.6 ng, epicotyl-170.0 ng, treated area-289.6 ng, hypocotyl-177.2 ng, roots-11.6 ng, nutrient solution-5.8 ng.

Effects of Constant Levels of GA and DCCD on Translocation of Various Levels of 2,4,5-T

At levels of 2,4,5-T from 0.5 µg to 5.0 µg, there is a geometric increase in the amount of auxin translocated from the treated area to

the young shoots (Table I). The ten-fold increase of auxin from 0.5 to 5.0 µg results in a 68-fold increase in auxin movement. This geometric increase is not observed in translocation to other areas of the plant; these show a more linear increase.

The 6.0 µg of DCCD with which some of the plants were treated did not alter the basic geometric aspect of translocation to the young shoots but it did decrease the magnitude. GA appeared to reduce the DCCD inhibition at all levels of auxin. However, at both the highest and the lowest levels of 2,4,5-T, 20.0 µg of GA added with the DCCD treatment did not significantly nullify the inhibition of translocation by DCCD.

Translocation of auxin to the primary leaves was reduced by DCCD at all levels of auxin. The GA significantly restored auxin movement to levels different from the DCCD-inhibited levels except for the 5.0 µg 2,4,5-T treatment level.

DCCD inhibited translocation of auxin to the epicotyl at the levels of 0.5 and 2.0 µg of 2,4,5-T, but not at the 5.0 µg level. Translocation at these lower two levels was partially restored by GA.

DCCD reduced by half or more, the amount of auxin translocated out of the treated area at all three auxin levels. Also, GA was effective in causing partial nullification of inhibition at all three levels.

As with the other parts of the plant, movement of auxin to the hypocotyl at the levels of 0.5 µg and 2.0 µg of 2,4,5-T was inhibited by the DCCD treatment. No significant inhibition occurred at the 5.0 µg level. GA restored translocation at the lowest auxin level to near that of the control. There appears to be a partial reversal of

TABLE I

EFFECTS OF GA AND DCCD ON TRANSLOCATION
OF VARIOUS LEVELS OF 2,4,5-T TO
DIFFERENT PARTS OF THE PLANT*

Treatment	Young Shoots	Primary Leaves	Epicotyl	Treated Area
0.5 µg 2,4,5-T	28a	280a	181a	363 a
0.5 µg 2,4,5-T+ DCCD	7b	112b	63b	825b
0.5 µg 2,4,5-T+ DCCD + GA	15b	196c	136c	596c
2.0 µg 2,4,5-T	462a	1166a	664a	1082a
2.0 µg 2,4,5-T+ DCCD	75b	447b	286b	3103b
2.0 µg 2,4,5-T+ DCCD + GA	272c	750c	517c	1971c
5.0 µg 2,4,5-T	1917a	2807a	1385a	2167a
5.0 µg 2,4,5-T+ DCCD	1172b	1661b	1126a	4790b
5.0 µg 2,4,5-T+ DCCD + GA	1578ab	2174ab	1289a	3266c

TABLE I (Continued)

Treatment	Hypocotyl	Roots	Nutrient Solution
0.5 µg 2,4,5-T	159a	24a	7a
0.5 µg 2,4,5-T+ DCCD	60b	5b	4b
0.5 µg 2,4,5-T+ DCCD+GA	119a	19ab	6ab
2.0 µg 2,4,5-T	665a	233a	102a
2.0 µg 2,4,5-T+ DCCD	246b	37b	23b
2.0 µg 2,4,5-T+ DCCD+GA	518ab	121b	45ab
5.0 µg 2,4,5-T	1474a	388a	204a
5.0 µg 2,4,5-T+ DCCD	1003a	366a	160a
5.0 µg 2,4,5-T+ DCCD+GA	1557a	414a	275a

*Levels of GA and DCCD were 20.0 µg and 6.0 µg per plant, respectively. Values are given in ng 2,4,5-T. Values for a single plant part and 2,4,5-T treatment followed by the same letter are not significantly different at the 5% level.

DCCD inhibition by GA at 2.0 µg of 2,4,5-T, but it is not statistically significant at the 95 percent level.

The accumulation of auxin in the roots and nutrient solution both showed inhibition by DCCD at the two lowest levels of auxin. GA did not significantly alter this inhibition although the trends for reversal were the same as for other plant parts.

Effects of Three Levels of DCCD and GA on Translocation of IAA

Interactions of three levels of GA (40.0 µg, 20.0 µg and 8.0 µg) with three levels of DCCD (8.0 µg, 4.0 µg and 1.6 µg) were investigated using a constant level of 0.165 µg of IAA (Table II). Virtually no differences were seen in IAA translocation at these different levels of DCCD or of GA. There was one exception. Acropetal auxin translocation to the primary leaves was significantly enhanced by high levels of DCCD over the low DCCD level. This enhancement was more extensive at low GA levels.

No other trends in auxin translocation such as were observed for the primary leaves were seen in other parts of the plant.

TABLE II

EFFECTS OF THREE LEVELS OF DCCD AND GA ON TRANSLOCATION OF IAA*

Treatment	Young Shoots	Primary Leaves	Epicotyl	Treated Area
IAA+40.0 µg GA+ 8.0 µg DCCD	4.9a	23.7ab	12.3a	124.7a
IAA+40.0 µg GA+ 4.0 µg DCCD	4.3a	19.6a	13.7a	130.7a
IAA+40.0 µg GA+ 1.6 µg DCCD	4.4a	16.9a	15.8a	131.2a
IAA+20.0 µg GA+ 8.0 µg DCCD	4.4a	35.2c	13.9a	124.5a
IAA+20.0 µg GA+ 4.0 µg DCCD	4.1a	29.2bc	16.2a	119.2a
IAA+20.0 µg GA+ 1.6 µg DCCD	5.1a	23.1ab	22.0a	118.5a
IAA+ 8.0 µg GA+ 8.0 µg DCCD	4.8a	65.9e	21.1a	122.4a
IAA+ 8.0 µg GA+ 4.0 µg DCCD	5.0a	43.4d	21.0a	124.5a
IAA+ 8.0 µg GA+ 1.6 µg DCCD	4.2a	23.6ab	19.6a	128.4a

TABLE II (Continued)

Treatment	Hypocotyl	Roots	Nutrient Solution
IAA+40.0 µg GA+ 8.0 µg DCCD	45.8a	20.0bc	3.2a
IAA+40.0 µg GA+ 4.0 µg DCCD	49.5a	23.4cd	3.3a
IAA+40.0 µg GA+ 1.6 µg DCCD	49.9a	20.4bc	3.3a
IAA+20.0 µg GA+ 8.0 µg DCCD	48.6a	16.3ab	3.8a
IAA+20.0 µg GA+ 4.0 µg DCCD	45.1a	18.8abc	3.2a
IAA+20.0 µg GA+ 1.6 µg DCCD	42.8a	20.8bc	2.8a
IAA+ 8.0 µg GA+ 8.0 µg DCCD	38.2a	17.7abc	3.0a
IAA+ 8.0 µg GA+ 4.0 µg DCCD	41.4a	13.7a	3.1a
IAA+ 8.0 µg GA+ 1.6 µg DCCD	49.6a	21.4bc	3.8a

*Each plant was treated with 0.165 µg IAA. Values are given in ng IAA. Values for a single plant part followed by the same letter are not significantly different at the 5% level.

CHAPTER IV

DISCUSSION

There was a definite inhibitory effect by DCCD on the translocation of 2,4,5-T in intact bean plants. The specific effect in the present experiments was one in which DCCD blocked the movement of 2,4,5-T from the site of application. In the presence of DCCD, 2,4,5-T did not enter the xylem and move in the translocation stream as was the case when other inhibitors such as cycloheximide was applied (Basler, unpublished data, 1977) nor did 2,4,5-T enter the living phloem and translocate to an appreciable extent in the assimilate stream. Apparently, the uptake of auxin into living cells must occur even before uptake into the xylem can occur when 2,4,5-T is placed in the pith tissue of the stem. DCCD is an inhibitor of ATPases (Harold et al., 1969). Thus, an ATPase activity may be coupled in some manner to the uptake of auxin into living cells or phloem such as might occur during vein loading. Similarly, workers have suggested or provided evidence that an ATPase may be involved in auxin action on growth (Hager et al., 1971). Kasamo and Yamaki (1976) observed that IAA stimulated activity of ATPase from membranes and that the activity was not due to <u>de novo</u> synthesis of the proteins. Marre et al. (1974) found that DCCD inhibited IAA and fusicoccin-induced growth and proton extrusion in pea internodes and noted that there seems to be an effect on an ATPase.

Where might the ATPase be located? Strong ATPase activity has been found on the plasma membranes of sieve tube elements and companion cells using a lead citrate stain (Yapa and Spanner, 1974). Hodges et al. (1972) have found ATPase activity in root membranes. This would correspond well with evidence that there is an auxin binding site on a plasma membrane-rich fraction of maize coleoptiles (Batt and Venis, 1976). However, Ray (1977) suggested that the major auxin binding site was located in the endoplasmic reticulum. Still, Belfagna et al. (1977) have shown that fusicoccin, a compound active in stimulating H^+/K^+ exchange in higher plants increases ATPase activity in vitro in plasma membrane fractions from maize coleoptiles and spinach leaves. Similarly, Kasamo and Yamaki (1976) showed by centrifugation fractionization procedures that a plasma membrane fraction was high in auxin binding capacity and that this was paralleled by high ATPase activity which was increased by prior auxin treatment. Thus, there is strong evidence that some property of auxin action is dependent on a membrane-bound ATPase.

In the present study, DCCD reduced translocation of 2,4,5-T to all parts of the plant. Normally, as the amount of 2,4,5-T injected into the plant is increased from 0.5 to 5.0 µg, the amount of auxin translocated to the growing points increased geometrically (Long and Basler, 1973). Above 5.0 µg of auxin, the translocation became linear. This may indicate that an auxin uptake or carrier site becomes saturated above 5.0 µg of 2,4,5-T. DCCD had no effect on the geometric aspect of translocation to the young shoots but it decreased the magnitude of the translocation. However, another translocation inhibitor, cycloheximide, did affect the shape of this translocation

curve. Cycloheximide caused the geometric increase to become a linear increase (Basler, unpublished data, 1977). Long and Basler (1973) also showed that cycloheximide reduced translocation of auxin to the roots and to the young shoots. However, in contrast to DCCD translocation, it increased translocation to the primary leaves. Translocation of auxin in injected bean seedlings from the treated area to the primary leaves probably occurs in the xylem (Long and Basler, 1973). This might indicate that cycloheximide causes efflux of auxin out of the living cells (possibly phloem) into the xylem, where it is carried in the transpiration stream up to the primary leaves. That cycloheximide also somewhat inhibits the epinastic response seen in bean seedlings injected with over 3.0 µg of auxin would also tend to support the conclusion that cycloheximide induces efflux of auxin out of the symplast.

What might be the mechanism of the geometric auxin response to the growing points? One possibility is that there is a Koshland-type positive cooperativity promoted by the auxin molecules that tends to cause more activation of the ATPase as more auxin is available. Another possibility, that might also account for the observed cycloheximide response, is that there is a negative cooperativity effect that inhibits auxin from effluxing from the phloem and entering the xylem thus providing more and more auxin to be available to be translocated upward into the phloem. The cycloheximide would cause this gate to be kept open, thus erasing the cooperativity which is normally expressed as the geometric increase in translocation. While the cycloheximide alters the translocation to a linear increase, translocation to young shoots in the presence of DCCD is still

geometric. This shows that the two compounds possibly act on different sites. Apparently, DCCD acts on the site of auxin uptake into living cells while cycloheximide acts on efflux. Other workers have found that auxin can move from phloem to the xylem with relative ease (Eliasson and Hallem, 1973, Zamski and Wareing, 1974).

Why is auxin not translocated geometrically to other parts of the plant? Possibly, this could be a mechanism to translocate the majority of the auxin to the area that needs it the most--the young, growing shoots. This would seem to indicate that the ATPase causing initial uptake toward the young shoots would have to be different from the one causing movement to the roots, for instance. Edwards and Hall (1974) have found a number of forms of ATPase in a single tissue, the root tips of maize. But perhaps the ATPase has nothing to do with it. Perhaps it is only in vascular tissue going to the young shoots that auxin has its effect on closing the "gate" through which auxin is leaked into the xylem.

There are a number of auxin activities that one must keep in mind in order to "get the whole picture" of auxin action. First, auxin must be synthesized at some point in the plant. Then it must be taken up by living cells (if it is not already in one) and transported in some fashion to its site of action. It must then enter the cell where it will, for instance, cause elongation of the cell walls. From that point, if it is to move in a polar manner, it must move from an individual cell to an adjacent cell. All of these possibilities have led to some confusion, making it difficult to conceptualize just how auxin effects its action. Must auxin move from outside to inside the cell to cause proton extrusion or does the auxin bind to the ATPase

from inside the cell? The evidence presently leads to no clear-cut conclusions.

Two theories of how protons are extruded during auxin-induced cell wall acidification are currently being investigated by a number of workers. One theory states that there is a simple exchange process of cations such as CA^{++} or K^{+} for protons inside the cell (Cohen and Nadler, 1976, Marre et al., 1974). During this process, electroneutrality is retained. The second theory holds that there is an electrogenic proton pump which is stimulated by auxin. This process would not rely heavily on the exchange of cations for protons, thus electroneutrality would not be retained (Cleland, 1977). In neither of these cases is the possibility of an ATPase excluded.

Just how auxin may act on or with an ATPase is not clear. Hager et al., (1971) suggested that auxin may act as an effector on an ATPase which would pump protons out of the cell as ATP is hydrolyzed to ADP and Pi. This could be correlated with the chemiosmotic theory of Mitchell (1974) in which a proton gradient is generated on one side of a membrane by hydrolysis of ATP on the other side. Another possibility might involve paired moving charges (Blondin and Green, 1975). In this theory, movement of the proton across the membrane might be coupled to movement of an electron or an anion. Transport through the membrane of an auxin molecule encapsulated in an ionophore may be associated with the action of the ATPase. DCCD might be involved by binding to the active site that auxin utilizes or by binding to an allosteric site that interferes with the binding or other activity of the auxin to the ATPase. Other evidence suggests that DCCD binds to the membrane at a site other than the ATPase because only membrane-bound

ATPase activity was affected by DCCD (Harold et al., 1969). In any event, the effect of DCCD tends to level off at higher DCCD concentrations indicating that there is an eventual saturation of binding sites.

Another aspect of this study was to investigate the effects of GA on the inhibition by DCCD of auxin translocation. GA was found to cause varying degrees of reversal of the inhibition by DCCD. This supports the findings of Katsumi (1976) who observed that GA reversed DCCD inhibition of natural and auxin-induced elongation of cucumber hypocotyls. GA may have its action by competing with DCCD for a site on the ATPase or the plasma membrane or by binding to a site allosteric to the DCCD site which would "protect" the enzyme from the DCCD. The reversal effect of GA doesn't tend to level off appreciably at the high levels used in this study. This may indicate that a high quantity of GA must be available to compete with DCCD lest the DCCD bind covalently and irreversibly to its auxin-inhibiting site.

Another possible explanation of the reversal could be that GA acts in some manner at the cell membrane to reduce the entrance of DCCD into the cell, thus preventing it from acting on the ATPase. The fact that GA reduces the adverse effects of other inhibitors as well as DCCD would lend credence to the idea that the action of GA may be broader than just an effect on the ATPase. Basler (1977) found that GA reversed the inhibitory effects of ancymidol and ethephon (growth retardants) on 2,4,5-T translocation to young shoots of bean plants. These results could allude to a physiological process in which endogenous GA protects the auxin translocation mechanism against endogenous growth inhibitors.

Another effect seen in some cases in this study was the increased basipetal translocation of auxin in plants treated with both DCCD and GA. This seems surprising in view of a previous report that GA alone reduced basipetal auxin translocation (Basler, 1974). No explanation of this additional effect of GA plus DCCD seems clear at this time.

In view of the possibility that DCCD and GA could be competing for a common site on the ATPase, an experiment using IAA as the auxin was designed to generate data for Lineweaver-Burk double reciprocal plots. Lineweaver-Burk plots are designed to distinguish between competitive and non-competitive inhibition between a substrate and an inhibitor for a site on an enzyme. However, using 0.165 μg of IAA, little difference in the effect on IAA by various levels of DCCD and GA was seen. In fact, since there was not a treatment without DCCD, it cannot be known whether the lowest concentration of DCCD was maximally inhibitory or whether there was no effect even with the highest level of DCCD. It may be that a relatively low level of DCCD such as used in the experiment could so saturate the auxin system at the low level of IAA used that active movement of auxin essentially ceased. However, it is possible that IAA translocation, in contrast to 2,4,5-T translocation, is not affected by DCCD. Thus, more work must be done to see if higher levels of IAA can be affected by DCCD. There was an effect of DCCD seen at the lowest level of GA on the promotion of translocation of IAA to the primary leaves. This movement, presumably through the xylem, could be due to the inhibition of IAA entrance into living cells by DCCD. The IAA, unable to enter living cells, could conceivably creep along the apoplast to the xylem, then on to the primary leaves.

In conclusion, auxin action seems to center around ATPases. Auxin transport (such as occurs in vein loading), also appears to depend in some manner on an ATPase. Therefore, the sites involved in auxin action may be the same or related to the site or sites involved in auxin transport.

CHAPTER V

SUMMARY AND CONCLUSIONS

Two aspects of auxin translocation in intact bean plants were studied: the inhibition of auxin translocation by DCCD and the reversal of the DCCD inhibition by GA.

The translocation of 0.5 µg of 2,4,5-T was inhibited by DCCD to all parts of the plant, especially at levels of DCCD above 6.0 µg per plant. Levels of GA above 6.0 µg per plant were found to reverse the inhibition of movement of 0.5 µg of 2,4,5-T caused by 6.0 µg of DCCD. This level of DCCD inhibited translocation of 2,4,5-T at levels of 0.5 µg and 2.0 µg of 2,4,5-T per plant but not at 5.0 µg.

There were no significant effects on translocation of 0.165 µg of IAA by levels of DCCD from 1.6 µg to 8.0 µg except in a single case of translocation to the primary leaves where auxin accumulation appeared to be enhanced rather than inhibited by DCCD. Levels of GA from 8.0 µg to 40.0 µg generally showed no trend in altering the effect of DCCD on IAA translocation.

The following conclusions are derived from this study.

DCCD causes a definite inhibition of translocation of 2,4,5-T in intact bean plants and GA causes varying degrees of reversal of this inhibition. These effects are not exhibited when using slightly lower levels of IAA in place of 2,4,5-T.

Since DCCD is an ATPase inhibitor, the evidence indicates that an ATPase may play a role in the uptake of 2,4,5-T into the living tissue (possibly phloem).

The possible mechanisms involved in (a) the geometric increase in auxin translocation to young shoots, (b) the inhibition of ATPase by DCCD and (c) the reversal of the DCCD inhibition by GA are discussed in Chapter IV.

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APPENDICES

APPENDIX A

RECIPE FOR HOAGLAND'S NUTRIENT SOLUTION

Add the following volumes of solutions to 24.625 l of distilled water:

1. 135 ml of 1 M $Ca(NO_3)_2$
2. 135 ml of 1 M KNO_3
3. 54 ml of 1 M $MgSO_4$
4. 27 ml of 1 M KH_2PO_4
5. 25 ml of Micronutrients

   To make micronutrient solution, add the following to distilled water to make 1 l:

   1. 2.86 g H_3BO_3
   2. 1.81 g $MnCl_2 \cdot 4H_2O$
   3. 0.11 g $ZnCl_2$
   4. 0.05 g $CuCl_2 \cdot 2H_2O$
   5. 0.025 g $Na_2MoO_4 \cdot 2H_2O$

APPENDIX B

RECIPE FOR Fe-EDTA SOLUTION

1. Dissolve 56.1 g of KOH in 1 l distilled water to make a 1 N KOH solution.

2. Take 279.8 ml of 1 N KOH and add distilled water to 500 ml. Heat to about 70 C and dissolve 26.2 g of EDTA (free acid).

3. Dissolve 24.9 g of $FeSO_4 \cdot 7H_2O$ in 300 ml of hot distilled water containing 4.0 ml of 1 N H_2SO_4.

4. Mix steps 2 and 3 and add distilled water to make 950 ml. Aerate vigorously for 12 hr.

5. Add distilled water to make 1 l. Contains 5 mg Fe/ml.

APPENDIX C

RECIPE FOR LIQUID SCINTILLATION COCKTAIL

For 2 l of cocktail, combine the following:

1. 160 g Naphthalene.
2. 10 g PPO.
3. 770 ml xylene.
4. 770 ml 1,4-Dioxane.
5. 464 ml ethanol.

VITA 2

Christopher William Corbett

Candidate for the Degree of

Master of Science

Thesis: THE INHIBITION BY N,N′-DICYCLOHEXYLCARBODIIMIDE OF TRANSLOCATION OF AUXIN IN INTACT BEAN SEEDLINGS AND ITS REVERSAL BY GIBBERELLIN A_3

Major Field: Botany

Biographical:

Personal Data: Born in Oklahoma City, Oklahoma, April 28, 1953, the son of Mr. and Mrs. T.M. Corbett.

Education: Graduated from Northeast High School, Oklahoma City, Oklahoma, in 1971; received the Bachelor of Science degree from Oklahoma State University, Stillwater, Oklahoma, in 1974; fulfilled requirements for Master of Science in December, 1977.

Experience: Graduate teaching assistant in School of Biological Sciences, 1975-1977.

Member: American Society of Plant Physiologists.